Comparative Inhibition Kinetics for Acetylcholinesterases Extracted from Organophosphate Resistant and Susceptible Strains of *Boophilus microplus* (Acari: Ixodidae)

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ABSTRACT In this study, acetylcholinesterases (AChEs) were extracted from two Mexican *Boophilus microplus* strains that demonstrated resistance to the organophosphate (OP) acaricide, coumaphos, in bioassay. The rate of inhibition of the extracted AChEs by the diethyl-OP paraoxon was determined for two resistant strains and two susceptible strains of *B. microplus*. The time to inhibition of 50% AChE activity was approximately two-fold greater for the resistant strains. Kinetic analysis of the interaction of the resistant AChEs with paraoxon revealed reduced bimolecular reaction constants (k_i). Apparent conformational changes in the AChE of the resistant strains were reflected in reduced K_m and V_{max} values. The bimolecular reaction constants (k_i) of the resistant strains were most affected by a slower rate of enzyme phosphorylation (k_2).

KEY WORDS acaricide resistance, acetylcholinesterase, AChE inhibition

THE SOUTHERN CATTLE tick, *Boophilus microplus* (Canestrini), is an important arthropod vector of bovine babesiosis (Smith and Kilborne 1893) and was eradicated from the United States in 1943 (Graham and Hourrigan 1977). The tick remains endemic to Mexico, and therefore, a threat for reintroduction into the United States. Reintroduction has been prevented by a surveillance and quarantine program maintained along the Texas–Mexico border. Cattle entering the United States from Mexico must be dipped in vats containing the organophosphate (OP) acaricide coumaphos (George 1996). Effectiveness of the quarantine program is dependent upon the efficacy of the acaricides used. Increasing reports of *B. microplus* resistance to pyrethroid and OP acaricides raise concern about continued effectiveness of the program (Santamaria and Fragoso 1994, Fragoso et al. 1995).

OPs and carbamates (CXs) are substrate analogues for acetylcholinesterase (AChE) and quasi-irreversibly inhibit AChE function (O'Brien 1967, p. 332; Fournier and Mutero 1994). AChE is associated with cholinergic synapses, and its normal function is essential for life (Fournier and Mutero 1994). Several mechanisms of OP resistance in arthropods are known: reduced penetration through the cuticle, detoxification and/or sequestration by metabolic enzymes, and target-site insensitivity (Villatte et al. 2000). The first modified AChE, demonstrating resistance to inhibition by OP, was associated with a resistant strain of the twospotted spider mite, *Tetranychus urticae* Koch (Smissaert 1964). The first report of AChE insensitivity in *B. microplus* was reported by Lee and Bantham (1966). AChE insensitivity is presently considered the principal mechanism of OP resistance in *B. microplus* (Bull and Ahrens 1988). However, the molecular basis for AChE insensitivity in *B. microplus* is unknown.

In this study, the kinetics of AChE inhibition by the diethyl-OP paraoxon of two Mexican OP-resistant strains and two OP-susceptible strains were compared. These kinetic characteristics describe the biochemical alterations that have occurred within these strains in the selection of insensitive AChEs. The molecular basis of these changes awaits the isolation of the AChE gene. Therefore this study is an initial step in elucidating the molecular basis of OP resistance in Mexican tick strains. Knowledge of that molecular basis offers various tools for use in the mitigation of OP resistance.

Materials and Methods

Tick Strains and Bioassay. Four *B. microplus* strains maintained at the Cattle Fever Tick Research Laboratory (CTFRL) in Mission, TX, were used in this study: two organophosphate susceptible and two organophosphate resistant strains. The susceptible strains include the Gonzalez and Munoz strains. The Gonzalez strain collected in 1994 and the Munoz strain collected in 1999 were obtained from outbreaks of ticks in Zapata County, TX. These susceptible strains have been under no laboratory acaricidal pressure. The resistant strains include the San Roman and the Caporal strains. The San Roman strain was collected in 1998 from a ranch (dairy herd) located in Champoton,

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Campeche, Mexico; and the Caporal strain was also collected in 1998 from a ranch 15 km from Champoton. The resistant strains have been under constant laboratory selective pressure, at the larval stage, with coumaphos (0.2–0.4% active ingredient).

Tick strains are routinely bioassayed with each generation by the personnel at the CFTRL using the FAO larval packet test (Stone and Haydock 1962), as described in detail by Miller et al. (1999). Probit analysis was run on bioassay results using Polo-PC to determine LD_{50} concentrations (LeOra Software 1987). Bioassay results were kindly provided by the CFTRL personnel.

Enzyme Extraction. Ticks (12–14-d-old-larvae, 0.1 g) were ground in 1 ml of tick extraction buffer (TEB) containing 10 mM sodium phosphate, pH 6.5, 20% sucrose, 1 mM EDTA, and 0.5% (v:v) Triton X-100, with a Ten Broeck tissue homogenizer (Kontes, Vineland, NJ). Proteins were extracted for 2 h at 4°C with gentle shaking. Solubilized protein was isolated by centrifugation at 14,400×*g* for 10 min in a Hermle Z 360 K centrifuge (National Labnet, Woodbridge, NJ) at 4°C. The supernatant was collected and recentrifuged at 14,400×*g* for an additional 3 min. The supernatant was again collected and the protein concentration determined with a Micro BCA method using bovine serum albumin as the standard (Pierce, Rockford, IL).

Enzyme Assay. AChE activity of larval tick extracts was measured with a modified Ellman assay (Ellman et al. 1961). The modified microplate assay used acetylthiocholine iodide (ASCh) as substrate. Sixteen μl of the enzyme preparation, which was made up of 8 μl of 50 mM sodium phosphate buffer (pH 7.5) and 8 μl of the enzyme extract containing 20 μg of total protein, was added to each plate well (6 replicates). A blank, without enzyme extract, was used as a control well. ASCh (200 μl) was added, and the reaction was monitored for 5 min, measuring absorbance at 405 nm at 1-min intervals. Substrate was prepared in 50 mM sodium phosphate buffer, pH 7.5, containing 0.32 mM Ellman's reagent, 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB, Sigma Chemical Co., St. Louis, MO). Kinetic constants (Michaelis constant, K_m; and maximum velocity, V_{max}) were determined with six concentrations of ASCh (120, 60, 30, 15, 7.5, and 3.75 μM). AChE activity was converted to moles of ASCh hydrolyzed per min by dividing the ΔOD/min by the molar extinction coefficient ($\epsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$). Kinetic constants were calculated with a rigorous nonlinear least squares method (Enzyme Kinetics Pro, ChemSW Software, Fairfield, CA) using the mean initial velocity (V_0) ± SD of replicates for each substrate concentration.

Inhibition Assay. The inhibition reaction in the presence of substrate and OP inhibitor is presented in the scheme.

$$E + PX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EPX \xrightarrow{k_2} EP + X$$

with the bimolecular reaction constant $k_i = k_2/K_d$, dissociation constant $K_d = k_{-1}/k_1$ and phosphorylation constant = k_2 and in the presence of substrate [S].

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{K_{cat}} E + \text{products}$$

In the scheme, E represents enzyme (AChE); PX, the OP with its leaving group; EPX, the reversible complex of enzyme and OP; and EP, the covalently phosphorylated enzyme (Hart and O'Brien 1973).

The diethyl-OP paraoxon [O,O-diethyl-O-(4-nitrophenyl) phosphate] (Sigma Chemical Co.), was used in this study to evaluate the rate of inhibition for AChE activity extracted from each strain. The rate of AChE inhibition for each larval tick extract (6 replicates/extract) was measured in the presence of 1.2×10^{-4} M ASCh and 20 μg tick extract. Paraoxon concentrations examined were 3.5×10^{-5}, 3.0×10^{-5}, 2.5×10^{-5}, 2.0×10^{-5}, 1.5×10^{-5}, and 1.0×10^{-5} M. The method used to determine the dissociation constant K_d, phosphorylation constant k_2, and the bimolecular reaction constant k_i was that of Chen et al. (2001). Briefly, the progressive inhibition of AChE activity at each inhibitor concentration was monitored over time (12 min, readings at 2-min intervals). The natural logarithm of the percent residual AChE activity with each concentration of paraoxon was plotted against the elapsed time and presented for demonstration in Fig. 1. The apparent rate constant (k), slope of the line, was determined by linear regression of the data points for each inhibitor concentration. The values for K_d, k_2, and k_i were determined by double reciprocal plots of apparent rate constants ($1/k$) against the inhibitor concentrations $\{1/[I](1-\alpha)\}$as demonstrated in Fig. 2 (Chen et al. 2001). The value for α was calculated by the equation $[S]/(K_m + [S])$.

Statistical Analysis. Data were analyzed by a one-way analysis of variance (ANOVA) (SigmaStat software, Kuo et al. 1992) with the Tukey test ($P < 0.05$) for pairwise comparisons except when data sets failed an equal variance test. Those data were analyzed by the Kruskal-Wallis one-way ANOVA on ranks with Dunn's method for pairwise comparisons ($P < 0.05$).

Results

AChE Activity. Analysis of AChE activity of protein extracts of resistant strains revealed they were both catalytically less active than AChE extracted from the susceptible strains ($F = 886.76$; df = 3,20; $P < 0.001$, Table 1). The San Roman strain ($V_{max} = 0.950 \times 10^{-6}$ moles/min/liter) had 67% of the activity of the susceptible Gonzalez strain ($V_{max} = 1.418 \times 10^{-6}$ moles/min/L), whereas the Caporal strain ($V_{max} = 0.538 \times 10^{-6}$ moles/min/L) was only 37.9% of the Gonzalez strain AChE activity. The K_m value for the Caporal strain was significantly lower than the susceptible strains and the San Roman strain, indicating an increased affinity of the enzyme for substrate ($F = 26.51$; df = 3, 23; $P < 0.001$). Although the K_m value for the San Roman strain was lower than the K_m values for

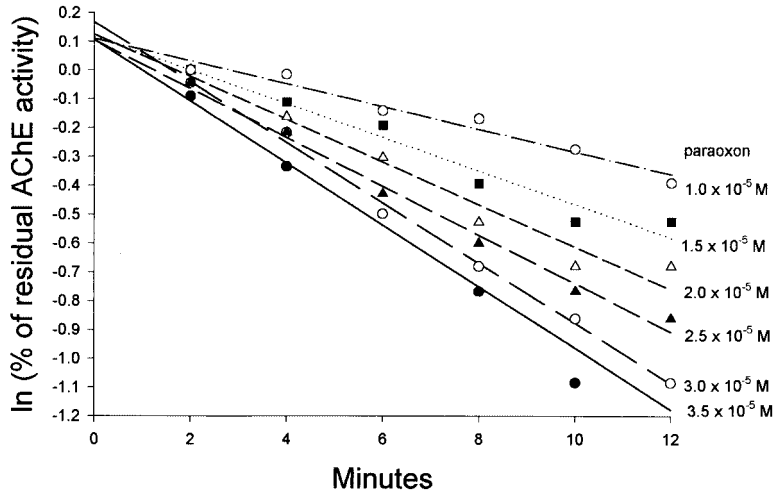

Fig. 1. Log-linear progression plot for the inhibition of AChE activity of *B. microplus* extract (San Roman, replicate 1) by increasing concentrations of paraoxon. These reactions were performed in the presence of 1. 2×10^{-4} M ASCh and 20 μg tick protein. The slope of the regression equation for each concentration was used as the apparent rate constant k for the construction of the double reciprocal plot $(1/k)$ to determine K_d, k_2, and k_i (Chen et al. 2001).

both susceptible strains, the difference was not statistically significant.

Inhibition Kinetics. The kinetic parameters of inhibition for the resistant and susceptible strains evaluated in this study are presented in Table 2. The K_d values, reflecting the affinity of the enzymes for OP, were not statistically different among the resistant and susceptible strains. The K_d values were not statistically different, due in part to variability as reflected in the large standard deviations. Statistically significant differences ($F = 5.26$; df = 3, 19; $P = 0.008$) were noted between the susceptible Munoz strain and resistant strains in the rate of phosphorylation (k_2). The rate of enzyme phosphorylation for both resistant strains was slower than for the susceptible strains. The inhibitory power, as measured by the bimolecular reaction constant (k_i), is dependent upon the affinity of the enzyme for OP (K_d) and the rate of phosphorylation (k_2) (Main 1964). Both resistant strains had k_i values significantly lower than the susceptible strains, further indicating their resistance to inhibition by paraoxon ($F = 40.76$; df = 3, 19; $P < 0.001$).

Time to 50% Inhibition. Resistance to paraoxon inhibition of AChE extracted from OP-resistant strains was evidenced by a prolonged time to 50% inhibition of AChE activity when in the presence of different

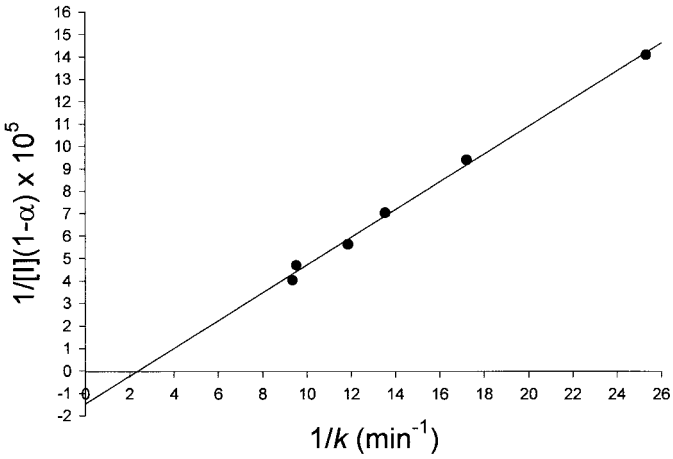

Fig. 2. Double reciprocal plot of rate of inhibition $1/k$ against the inhibitor concentration $1/[\mathrm{I}](1-\alpha)$ (Chen et al. 2001). Presented for example is replicate 1 of San Roman extract, intercept of x-axis yields the phosphorylation constant k_2 (1/2.374 min = 0.4211 min^{-1}), intercept of y-axis yields the dissociation constant K_d ($1/1.4672 \times 10^5 = 6.8157 \times 10^{-6}$ M), and the slope of the line ($0.6178 = 6.178 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}$, $r^2 = 0.996$) yields the k_i, or $k_i = k_2/K_d$.

Table 1. Kinetic constants of extracted *B. microplus* AChE

Strain (generation)	$K_m \pm$ SD $\times 10^{-6}$ M	$V_{max} \pm$ SD $\times 10^{-6}$ moles/min/liter
Gonzalez (27)	9.616 ± 0.920a	1.418 ± 0.042a
Munoz (9)	9.420 ± 1.117a	1.687 ± 0.065b
Caporal (7)	5.881 ± 0.436b	0.538 ± 0.008c
San Roman (11)	9.181 ± 0.745a	0.950 ± 0.030d

Values in columns with different letters are significantly different ($P < 0.05$).

concentrations of paraoxon (Table 3). At each concentration of paraoxon, the time to 50% inhibition of AChE activity was approximately doubled for the resistant strains.

Discussion

Acetylcholinesterase is of interest because it is the target-site for organophosphate and carbamate pesticides in the central nervous system, and its role in cholinergic synapses is essential for life (Fournier and Mutero 1994). Although metabolic and potential sequestration mechanisms of OP resistance have been experimentally demonstrated in the tick (Roulston et al. 1969, Bull and Ahrens 1988, Miller et al. 1999, Jamroz et al. 2000), AChE insensitivity is considered the principal resistance mechanism that leads to acaricide product failure (Lee and Bantham 1966, Schuntner et al. 1968, Roulston et al. 1968, Bull and Ahrens 1988, Wright and Ahrens 1988). AChE insensitivity in OP resistant *B. microplus* ticks has been experimentally demonstrated. Evidence of reduced AChE activity associated with resistant ticks has been reported (Lee and Bantham 1966, Nolan et al. 1972, Wright and Ahrens 1988). Investigators have demonstrated that increased concentrations of OP are required to inhibit 50% of AChE activity in resistant tick strains (Roulston et al. 1968, Wright and Ahrens 1988). They have also demonstrated slower rates of AChE inhibition in resistant tick strains as determined by the measurement of k_i (bimolecular rate constant) by the method of Aldridge (1950) in the absence of substrate (Lee and Bantham 1966, Roulston et al. 1968, Nolan et al. 1972).

A molecular basis for target site insensitivity suggests that point mutations within the AChE gene (*Ace*) result in amino acid substitutions that alter AChE conformation and affinity for OP (Morton 1993, Fournier and Mutero 1994). AChE resistance to OP has been demonstrated to be associated with five amino acid replacements in *Drosophila melanogaster* (Meigen) with several of the mutations simultaneously present in the same gene (Mutero et al. 1994). The mutations appearing singularly showed slight resistance levels, but in combination increased resistance levels (Fournier et al. 1992; Fournier and Mutero, 1994). Thus, genetic recombination of point mutations with weak individual contributions to resistance into the same gene can lead to increased target site insensitivity (Mutero et al. 1994). Chen et al. (2001), in an elegant experiment, used site-directed mutagenesis to create the same *Ace* point mutations found in *D. melanogaster* within the *Ace* homologue of *Lucilia cuprina* (Wiedemann). These mutations were introduced singularly and in combination and expressed in a baculovirus system to characterize their kinetic properties and interaction with OP. The single and combined mutations altered the affinity of the AChE for OP and increased target site insensitivity (Chen et al. 2001).

In this study, a significant reduction in V_{max} values was observed for the resistant tick strains. This loss of AChE activity is consistent with increased insensitivity in most cases (Fournier and Mutero 1994), although there have been cases of unaltered (Zhu and Brindley 1990) or increased AChE activity in resistant strains (Plapp and Tripathi 1978). In addition, both resistant tick strains had lower K_m values than did the susceptible strains indicating alterations in their AChEs that has resulted in an increase in affinity for substrate. Although lower, the K_m value for the San Roman strain was not significantly lower than for the susceptible strains; however, the K_m value for the Caporal strain was significantly lower than both susceptible strains. Conformational alterations in AChE that affect affinity for substrate can result in either a higher K_m reflecting decreased affinity (Fournier & Mutero 1994) or a lower K_m reflecting increased affinity (Morton and Singh 1982). Changes in affinity of the enzyme for substrate generally result in lower V_{max} values. Rate of product formation could be decreased as a result of reduced affinity (higher K_m) of enzyme for substrate, as the rate of the enzyme-substrate complex would be affected, and the rate of enzyme acetylation could be affected by an increase in the affinity of enzyme for substrate (decreased K_m) (Fournier and Mutero 1994).

Table 2. Dissociation constants (K_d), phosphorylation constants (k_2), and bimolecular reaction constants (k_i) for AChE in extracts of *B. microplus* larvae

Strain (generation)	$k_2 \pm$ SD (min^{-1})	$K_d \pm$ SD ($\times 10^{-6}$ M)	k_i SD ($\times 10^5$ M^{-1})	LC_{50} (%AI)[a]
Gonzalez (27)	0.896 ± 0.602ab	6.134 ± 4.737a	1.564 ± 0.240a	0.024 (25)
Munoz (9)	1.412 ± 0.764a	10.088 ± 6.484a	1.481 ± 0.174a	0.023 (8)
Caporal (7)	0.291 ± 0.067b	2.536 ± 0.855a	1.179 ± 0.125b	0.428 (8)
San Roman (11)	0.527 ± 0.317b	9.099 ± 6.353a	0.609 ± 0.069c	0.354 (11)

Values in columns with different letters are significantly different ($P < 0.05$). Strain susceptibility to coumaphos, as defined by bioassay results, presented in column labeled lethal concentration 50% (LC_{50}).

[a] Lethal concentration 50% (LC_{50}) is presented as % active ingredient (AI) of the diethyl-OP coumaphos, number in parenthesis represents the generation bioassayed.

Table 3. Time (minutes ± SD) to inhibition of 50% AChE activity in the presence of various concentrations of paraoxon

Strain (generation)	Paraoxon concn.[a]					
	3.5×10^{-5} M	3.0×10^{-5} M	2.5×10^{-5} M	2.0×10^{-5} M	1.5×10^{-5} M	1.0×10^{-5} M
Gonzalez (27)	3.275 ± 0.283a	3.833 ± 0.170a	4.428 ± 0.119a	5.470 ± 0.408a	6.456a	8.647a
Munoz (9)	3.370 ± 0.236a	4.188 ± 0.250b	4.537 ± 0.522a	5.717 ± 0.193a	6.128a	8.981a
Caporal (7)	6.257 ± 0.348b	6.829 ± 0.228c	7.344 ± 0.556b	8.926 ± 0.451b	11.115ab	15.217ab
San Roman (11)	7.794 ± 0.407c	8.203 ± 0.068d	9.244 ± 0.245c	10.994 ± 0.362c	13.865b	21.286b

Paraoxon present in the reaction with 1.2×10^{-4} M ASCh and 20 μg tick protein. Progression of inhibition was monitored at 2 min intervals for 12 min. The natural logarithm of the % residual activity was plotted against the elapsed time. A line was regressed between those data points and the times to 50% inhibition extrapolated from the regression equation. Values in columns with different letters significant at $P < 0.05$. Data analyzed by a one-way ANOVA with the Tukey test for pairwise comparisons except for data in 1.5×10^{-5} M and 1.0×10^{-5} M columns. Due to the failure of an equal variance test, those data were analyzed by the Kruskal-Wallis one-way ANOVA on ranks with Dunn's method for pairwise comparisons with median values presented.

[a] Paroxon concentrations: 3.5×10^{-5} M, ($F = 264.79$; df = 3, 19; $P < 0.001$); 3.0×10^{-5} M, ($F = 722.23$; df = 3, 19; $P < 0.001$); 2.5×10^{-5} M, ($F = 198.72$; df = 3, 19; $P < 0.001$); 2.0×10^{-5} M, ($F = 270.57$; df = 3, 18; $P < 0.001$); 1.5×10^{-5} M, ($H = 18.92$; df = 3; $P = < 0.001$); 1.0×10^{-5} M, ($H = 19.06$; df = 3; $P < 0.001$).

An analysis of inhibition kinetics revealed no statistical difference between the resistant and susceptible strains relevant to the affinity of AChE for OP (K_d). However, AChE extracted from the Caporal strain did have the lowest K_d value of the strains evaluated. This observation coupled with a significant decrease in the K_m value for ASCh for the Caporal strain would suggest that Caporal strain AChE has increased affinity, relative to the other strains, for ASCh and OP, a substrate analogue. This is somewhat unique because most mutations within the *Ace* gene have appeared to reduce affinity and in effect increase K_d, for the OP (Chen et al. 2001). Of significance in this study was the reduced rate of phosphorylation (k_2) observed in the resistant strains. This slower rate resulted in significantly lower bimolecular reaction constants (k_i) for the resistant strains indicative of target-site insensitivity. Like the observed increase in substrate affinity, these observations of increased affinity for OP and reduced phosphorylation rates are unique. Resistance is often associated with an increase in K_d reflecting a loss of affinity for the toxicant (Fournier and Mutero 1994), although a decrease in the rate of phosphorylation is not without precedent (Smissaert et al. 1970). The kinetic results of this study and the increased insensitivity of the AChEs from the resistant strains are further supported by the time required to inhibit 50% of AChE activity (Table 3). The decrease in the rate of inhibition is reflected in that it takes approximately twice the time to inhibit 50% of AChE activity in the resistant strains compared with the susceptible strains.

These biochemical results strongly suggest that mutational events have occurred in the gene encoding *B. mircoplus* AChE. However, they do not allow for the identification of the resistance mutations. This can only be accomplished by isolating the *B. microplus* AChE gene. Baxter and Barker (1998) and Hernandez et al. (1999) have isolated putative cDNA clones of AChE from *B. microplus.* However, no point mutations were detected in the gene coding regions from OP resistant strains of *B. microplus.* Baxter and Barker (1998) suggested that alterations in AChE conformation occur post-translationally or that there is another undefined AChE locus.

In this study, apparent allelic differences have been demonstrated biochemically in Mexican *B. microplus* resistant strain AChEs. These differences account for variation in increased affinity for substrate, OP, and reduced rates of phosphorylation relative to susceptible strains. Therefore, future focus of this work is to isolate the relevant AChEs from each strain and obtain amino acid sequence information allowing for the isolation of the respective genes. A comparison of those gene sequences with the sequence reported by Baxter and Barker (1998) may further elucidate the conundrum about additional loci and/or post-translational modification. Knowledge of the molecular basis of resistance allows for the development of molecular diagnostic probes, management of genes within tick populations, and possible reversion of resistance with antiresistant compounds (Fournier and Mutero 1994).

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